Chlorantraniliprole (Rynaxypyr): A novel DuPont™ insecticide with low toxicity and low risk for honey bees (*Apis mellifera*) and bumble bees (*Bombus terrestris*) providing excellent tools for uses in integrated pest management

Axel Dinter[1]*, Kristin E. Brugger[2], Niels-Martin Frost[3], Michael D. Woodward[2]

[1]DuPont de Nemours Deutschland (GmbH), DuPont Str. 1, D-61352 Bad Homburg v.d.H., Germany.
[2]DuPont Crop Protection, Stine-Haskell Research Center, 1090 Elkton Road, Newark, DE 19714, USA
[3]DuPont Danmark ApS, Sköjtevej 26, DK-2770 Kastrup, Denmark
*Tel. +49(0)6175797211, Email: axel.dinter@dupont.com.

Abstract

Background: The effects on bees of chlorantraniliprole (DPX-E2Y45, DuPont™ Rynaxypyr), a new anthranilic diamide insecticide with a novel and very specific mode of action activating insect ryanodine receptors were investigated.

Results: Acute toxicity tests with chlorantraniliprole and the formulations, Coragen and Altacor, demonstrated low intrinsic toxicity to honey bees. Low risk for honey bees was demonstrated in semi-field tunnel tests with flowering *Phacelia* or wheat (with daily sprays of sugar solution to simulate honey dew) at application rates of Coragen of up to 60 g chlorantraniliprole/ha. Low potential of systemic exposure via pollen and nectar of honeybees to chlorantraniliprole was documented in a residue *Phacelia* tunnel trial with chlorantraniliprole applied to and mixed into bare soil. The impact of Altacor on bumble bees was studied in a greenhouse test in tomato at 40 g chlorantraniliprole/ha. Bumble bees directly over-sprayed during foraging activity with chlorantraniliprole or exposed to treated plants behaved as controls.

Conclusion: Chlorantraniliprole formulations provide excellent tools for integrated pest management (IPM) programmes to conserve pollinating honey bees and bumble bees.

Keywords: Chlorantraniliprole, Rynaxypyr®, insecticide, side-effects, honey bee, bumble bee, integrated pest management (IPM)

Introduction

Chlorantraniliprole (DuPont™ Rynaxypyr®) is a new anthranilic diamide insecticide developed worldwide by E.I. du Pont de Nemours and Company, Inc. with a novel and very specific mode of action. Chlorantraniliprole activates ryanodine receptors via stimulation of the release of calcium stores from the sarcoplasmic reticulum of muscle cells (i.e. for chewing insect pests) causing impaired regulation, paralysis and ultimately death of sensitive species[1]. The differential selectivity chlorantraniliprole has towards insect ryanodine receptors explains the outstanding profile of low mammalian toxicity[2]. Chlorantraniliprole is active on chewing pest insects primarily by ingestion and secondarily by contact. In Europe, Coragen® and Altacor® have been developed for foliar applications in top fruit, vegetable crops, grapes and potatoes at rates of 10 to 60 g chlorantraniliprole/ha, which are highly effective on many important pest insects[3]. The chlorantraniliprole formulations, Coragen and Altacor, were demonstrated to have negligible effects on numerous beneficial non-target arthropod species (e.g. the predatory mite *Typhlodromus pyri* or the parasitic wasp *Aphidius rhopalosiphi*) or to have rather low and transient impact on some slightly sensitive beneficial species[4]. This paper summarizes the current knowledge on effects of chlorantraniliprole and the formulated products, Coragen and Altacor, on honey bees and bumble bees.

Experimental methods

Effects of the active substance, chlorantraniliprole (also known as DPX-E2Y45 or Rynaxypyr), and two formulations, Coragen (200 g Rynaxypyr/L; DPX-E2Y45 20SC) and Altacor (350 g Rynaxypyr/kg; DPX-E2Y45 35WG), were studied using adopted test guidelines for honey bees (e.g. OECD or EPPO or CEB test methods) or with some modifications to address specific questions.

Acute honey bee testing

The intrinsic toxicity of the active substance chlorantraniliprole to the honeybee (Apis mellifera L.) (Hymenoptera, Apidae) was investigated in an acute oral and contact test following OECD Guideline No. 213 and No. 214[5,6]. Chlorantraniliprole is characterised by low solubility in water with a maximum solubility of 1 mg chlorantraniliprole/L water at 20°C. In the contact test, a stock solution of chlorantraniliprole was prepared in water at 1 mg active substance/L and either one 2-µL-droplet or one 5-µL-droplet were applied on the dorsal thorax of each honey bee to achieve maximal nominal doses of 2 and 5 ng chlorantraniliprole/bee. In the oral test – using the same water dilution approach – the bees were exposed to a dose of 27.4 ng chlorantraniliprole/bee. In another test acetone as an organic solvent was used to allow oral and contact testing at higher doses knowing that acetone is not used as an inert in any DuPont™ Rynaxypyr formulations. Oral and contact tests with the formulated products were performed without the use of any additional organic solvents.

Semi-field tunnel honey bee testing to assess effects from spray application

Coragen was chosen as the test substance for assessment of potential effects of chlorantraniliprole formulations under worst-case semi-field conditions because some sub-lethal effects were observed in the acute tests for this formulation, while no behavioural effects were observed for the Altacor formulation. Semi-field tests with small honey bee colonies that contained all brood stages at test start assessed the following effects during the pre- and post-application period: A) Mortality (counts of the numbers of dead honey bees in front of the hive and on sheets on the soil surface within the tunnel tests pre- and post-treatment), B) Foraging activity (visual counts of the numbers of foraging honey bees/m²), C.) Behavioural effects (visual assessments of the behaviour of the foraging honey bees on the crop and of honey bees around the hive), D.) Brood effects (assessments of the status of the honey bee colony regarding visibility of the honey bee queen and availability of eggs, larvae, pupae and adult honey bees inside the hive). Protocols fulfilled test guideline criteria although some observations were made at greater frequency than specified in guidelines in order to characterize potential changes as closely as possible.

Semi-field tunnel honey bee tests according to eppo 170-3: Three semi-field tunnel tests were conducted following the EPPO 170-3 test design with flowering *Phacelia tanacetifolia* Benth. as a model crop[7]. One trial each was performed in Germany and Spain with the formulated product Coragen and an application rate of 52.5 g chlorantraniliprole/ha. A third trial was conducted in France with the formulated product Coragen and an application rate of 60 g chlorantraniliprole/ha. The spray applications were all performed with hand-held boom sprayers at 400 L spray volume/ha during full flowering of the *Phacelia* crop and during foraging activity of the honey bees. Each trial had nine tunnels, three separated tunnels each for the control, chlorantraniliprole and toxic standard (260 g dimethoate/ha) treatments. Tunnels comprised an area of 50 to 60 m²/tunnel.

Semi-field tunnel honey bee tests according to ceb 230: Six semi-field tunnel tests following the CEB 230 test design were performed with flowering *Phacelia tanacetifolia* and winter winter wheat as model crops[8]. Three separate trials for each crop were performed in France with the formulated product Coragen and an application rate of 60 g chlorantraniliprole/ha. The spray applications were all conducted with hand-held boom sprayers and 200 or 300 L spray volume/ha during full flowering of the *Phacelia* crop or after wheat was sprayed with sugar solution to simulate honey dew. The control, one chlorantraniliprole and the toxic reference treatment (260 or 400 g dimethoate/ha) were sprayed while the honey bees were foraging, while another chlorantraniliprole tunnel was sprayed either in the late evening after daily honey bee flight or early in the morning before daily honey bee flight. There was one tunnel for each for the four treatments in line with the test guideline comprising an area of 64 to 80 m²/tunnel.

Semi-field tunnel honey bee test to quantify residue in bee matrices via systemic uptake from the soil

A semi-field study was conducted according to EPPO 170-3 and focused on residue analyses to determine whether chlorantraniliprole residues carried over in soil after applications at planting to the nectar or pollen in future flowering crops and to compare to the residues of an application made when bees were foraging[7].

Phacelia tanacetifolia was used a model crop because it is highly attractive to honey bees and is a fast-growing plant species with intensive roots growing in the top soil layer that was dosed with chlorantraniliprole. Residues of chlorantraniliprole were quantified in pollen and stomach nectar from foraging bees that returned to the hive, as well as residues in pollen, nectar and wax inside the hive after at-plant soil applications (to simulate soil residues from carry over) or foliar application. Soil was dosed at a rate that would simulate a long-term plateau concentration resulting from continuous maximum use over multiple years.

Treatments – with two separate tunnels each comprising each a crop area of about 100 m² – consisted of (a) a tap water control (C), (b) Coragen applied at 253.6 g chlorantraniliprole/ha and incorporated into the soil (10 cm depth) on the day of sowing of *P. tanacetifolia* followed by a second application at 60 g chlorantraniliprole/ha to the soil surface after sowing (equivalent to the estimated maximum soil exposure at the time of the study conduct) (T1) and (c) Coragen applied once at 60 g chlorantraniliprole/ha onto flowering *P. tanacetifolia* while honey bees were foraging (equivalent to worst-case exposure during foraging activity) (T2). Analytical dose verification in the soil demonstrated correct soil incorporation with chlorantraniliprole.

Forager bees were collected on 4 sampling days during exposure to flowering *P. tanacetifolia*. The samplings in all 3 treatments (C, T1 and T2) were conducted once before application of T2 and control (DAA-1; DAA = Days after application)) and three times after application of T2 and control: DAA+1, DAA+4 and DAA+7 (days after application). The bees were frozen (≤-18 °C) until the preparation of the honey stomachs and pollen loads from the forager bees and residue analysis. The pollen, nectar and wax samples (from the combs) were collected once before (DAA-1) and two times after application of T2 and control (DAA+1 and DAA+7). Each comb sample was taken from 3 spots per hive. For the sampling, pieces of combs with pollen and nectar were cut out from the combs by using a clean knife for each sample. During the assessment days it was tried to assure that the pollen and nectar collected was fresh collected from the *P. tanacetifolia* plot. The comb pieces for collecting pollen, nectar and wax were stored deep-frozen within 6 h after sampling (≤-18 °C) until residue analysis.

Bumble bee greenhouse testing

The objective of the study was to determine the effects of the insecticide Altacor on the bumble bee *Bombus terrestris* L. (Hymenoptera, Apidae) under semi-field conditions (greenhouse) in tomato based on general SETAC/ESCORT and EPPO 170-3 recommendations[7,9].

Young normal queen-right colonies each with 25 worker bumble bees were used. The colonies were matched for similar amounts of brood (larvae and pupae) at various stages of development. The colonies for the test were set-up in the greenhouse on 26 October 2007 in the late afternoon. The application of Altacor was performed in the greenhouse with flowering tomato plants. No bumble bee hives were used by the farmer before the introduction of the test hives. Four Altacor treatment and a control group were investigated: T1 = Altacor applied during foraging activity of the bumble bees, T2, T3 and T4 = Altacor applied 24 h, 48 h and 72 h before opening the hives, respectively; T2, T3 and T4 were applied with closed bumble bee hives and no bumble bees in the plots. On the day of the application in T1 and the control the bumblebees of all treatments were exposed (= start of exposure). After start of exposure the colonies were kept for 21 days in the greenhouse and assessed for mortality, foraging activity, condition of colonies and development of bumble bee brood. In each of the test item treatments the application of Altacor was performed at a rate of 114.3 g Altacor/ha (equivalent to 40.0 g chlorantraniliprole/ha) and at a target application volume of 1000 L/ha. Each treatment group comprised 4 greenhouse plots of at least 420 m^2 with one bumble bee hive/plot. The plots were separated by a net with a maximum mesh size of 5 mm. The study was located in Mazarron, region Murcia, Spain. The influence of Altacor was evaluated by comparing the results in the four Altacor treatments to the control regarding the following observations: Number of living and dead worker bees and larvae, foraging activity as measured by flower visits (bite marks), consumption of sugar solution, development of the bumble bee brood and condition of the colonies. The tomato blossoms were classified in 4 categories and each category received points (category 1: no bite mark = 1 point; category 2: 1-3 bite

marks/blossom = 2 points; category 3: > 3 bite marks/blossom = 3 points; category 4: blossom with brown pistil = 4 points).

Results

Acute honey bee toxicity

Low intrinsic honey bee toxicity of the active substance chlorantraniliprole and both demonstrated products, Altacor and Coragen, was demonstrated in acute oral and contact tests (Table 1). When the active substance chlorantraniliprole was tested up to the maximum solubility in water no significantly increased mortality or any sub-lethal effects of the honey bees were observed compared to the controls. The oral and contact LD_{50} values using water as solvent were >0.027 and >0.005 µg chlorantraniliprole/bee, respectively. Using acetone as organic solvent, which is not an actual inert in any of the DuPont chlorantraniliprole formulations, the oral and contact LD_{50} values were >104 and >4 µg chlorantraniliprole/bee, respectively. Under these artificial test conditions the honey bees were lethargic or apathetic following dosing but recovered during the following 48 to 72 h. Formulation testing did not require the use of any additional solvents. For Altacor the oral and contact LD_{50} value were >119.2 and 100 µg chlorantraniliprole/bee, respectively and no sub-lethal effects were observed at any dose tested. For Coragen the oral and contact LD_{50} value were >117.8 and 81.5 µg chlorantraniliprole/bee, respectively. Some honey bees treated with Coragen showed sub-lethal effects at the highest dosages tested. The oral and contact NOEC determined for Coragen were 63 and 12.5 µg chlorantraniliprole/bee, respectively. The hazard quotients (HQ) for both formulated product assuming the worst-case EU label rate of 60 g chlorantraniliprole/ha for any chlorantraniliprole formulation were all less than much less than one.

Table 1 Acute oral and contact toxicity of chlorantraniliprole and formulated products on honey bees and hazard quotients (HQ) [For the calculations of the HQs – defined as the maximum single application rate in g/ha divided by the LD50 in µg a.s./bee – the worst-case EU label rate of any chlorantraniliprole formulation of 60 g chlorantraniliprole/ha was considered].

Test material	**Oral LD_{50} (µg chlorantraniliprole per honey bee)**	**Contact LD_{50} (µg chlorantraniliprole per honey bee)**	**HQ_{oral}**	**$HQ_{contact}$**
Chlorantraniliprole (in water)	>0.027	>0.005	<2190	<12000
Chlorantraniliprole (in acetone)	>104	>4	<0.6	<15
Altacor	>119.2	>100	0.5	0.7
Coragen	>117.8	>81.5	<0.5	0.6

Results of semi-field tunnel honey bee tests to assess effects of spray application

Results of semi-field tunnel honey bee tests according to EPPO 170-3: Three fully replicated honey bee tunnel tests were conducted with spray application of Coragen at either 52.5 g (trials in Germany and Spain in 2004) or 60 g chlorantraniliprole/ha (trial in France in 2006). Results are summarized in Table 2. As an example, only the results of the trial with the highest application rate will be described in detail. On the day of application just before spray application high foraging activity was visually assessed with about 17 honey bees/m² in all three treatment groups. The foraging activity in the control and the Coragen treatment continued to be high following the spray application and was found to be >20 honey bees/m² the following day. Over the whole 7-day post-treatment assessment period no remarkable differences between the numbers of foraging honey bee/m² in the control and Coragen treatment was found, while in the toxic reference the numbers were low or zero (Figure 1).

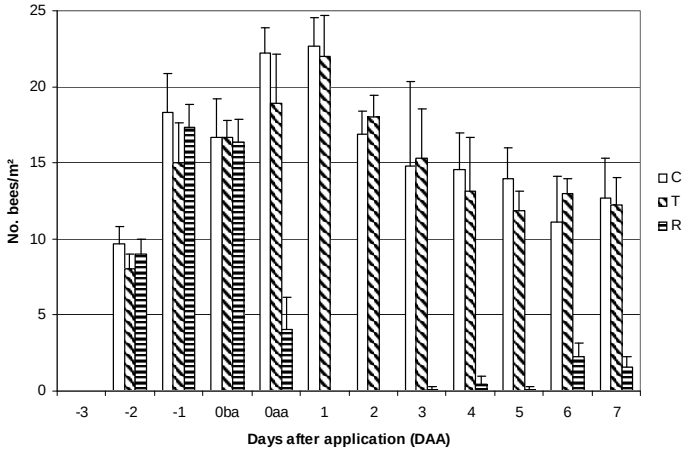

Figure 1 Mean honey bee flight intensity (number of honey bee/m² ± SD) in the control (C), Coragen at 60 g chlorantraniliprole/ha (T) and toxic reference treatment (dimethoate) prior to and after spray application during honey bee foraging activity in flowering *P. tanacetifolia* in France, 2006. (0ba = evaluation on the day of treatment shortly before application; 0aa = evaluation on the day of treatment after application)

During the pre-application period comparable numbers of dead honey bees were determined in all 3 treatment groups. On the day of treatment before application a mean number of 19.7 dead honey bees/tunnel was observed in the Coragen group compared to 22.0 dead honey bees/tunnel in the control and in the reference item treatment group, respectively. On the same day after application the mean number of dead bees in the Coragen treatment group was 24.7 dead honey bees/tunnel. In the control treatment a mean number of 12.7 dead honey bees/tunnel was found, while in the reference treatment group the mean number of dead honey bees increased to 605.0 dead honey bees/tunnel (Figure 2).

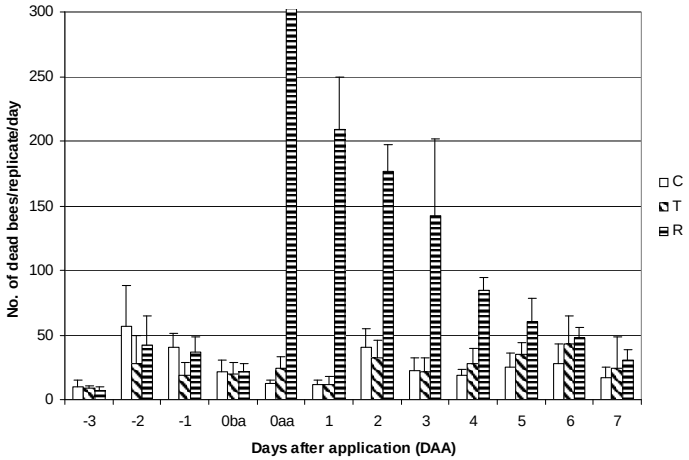

Figure 2 Mean number of dead honey bees/replicate tunnel/day (± SD) in the control (C), Coragen at 60 g chlorantraniliprole/ha (T) and toxic reference treatment (dimethoate) prior to and after spray application during honey bee foraging activity in flowering *P. tanacetifolia* in France, 2006. (0ba = evaluation on the day of treatment shortly before application; 0aa = evaluation on the day of treatment after application)

The mean post-application mortality was determined to be 27.7 dead honey bees/tunnel/day in the Coragen treatment group compared to 22.1 dead honey bees/tunnel/day in the control group and 169.5 dead honey bees/tunnel/day in the reference item treatment group. No significant differences were determined between pre-application mortality values of the Coragen, reference and control treatment (t-Test or Mann-Whitney test, $p > 0.05$). Furthermore no significant differences were found between pre- and post-application mortality data of the Coragen treatment group and the control group (t-Test, $p > 0.05$). The post-application mortality of the reference group was significantly different compared to the control group as well as of the Coragen treatment group (t-Test, $p < 0.05$). At the brood assessments carried out once before exposure (DAA-4) and 4-times after treatment (DAA+7, DAA+14, DAA+22 and DAA+28) all brood stages (egg stage, larval and pupal stage) in the colonies of all treatment groups were available. There were no differences between assessments of the strength of the colonies (number of bee ways between combs filled with honey bees) in the Coragen treatment group and control. The colonies of the control and Coragen treatment groups showed neither in the pre- nor in the post-application period noteworthy abnormal behaviour. In the toxic reference group abnormal honey bee behavior (cramping; collecting at the entrance) was noticed on the day of application after the spray application.

The results of all three EPPO honey bee tunnel trials with *P. tanacetifolia* are summarized in Table 2. Generally, honey bees resulted in mortality at levels comparable to the control group following exposure to Coragen spray solutions by direct overspray onto foraging honey bees. Also, there were no obvious differences found between the control and the Coragen treatment group regarding flight intensity, behaviour, colony strength or presence of queen, eggs, larvae or pupae.

Table 2 Results of three semi-field honey bee tunnel test according to EPPO 170-3 with Coragen sprayed during honey bee foraging activity in flowering *P. tanacetifilia*. (DAA = Day after spray application)

Country Year Rate	**Mortality Flight intensity Behaviour**	**Colony health (Hive assessment regarding colony strength and presence of queen, eggs, larvae and pupae)**
Germany 2004 52.5 g a.s./ha	No significant increase in mortality and no inhibition of flight intensity and no changes in individual behaviour compared to control	Colony strength not affected versus control All brood stages present on DAA+8
Spain 2004 52.5 g a.s./ha		Colony strength not affected versus control All brood stages present on DAA+22
France 2006 60 g a.s./ha		Colony strength not affected versus control All brood stages present on DAA+7, +14, +22, +28

Results of semi-field tunnel honey bee tests according to CEB 230: As an example for the three CEB trials conducted with wheat and daily sprays with sugar solution and simulate honey dew the results of a study conducted in 2006 in western France will be described. The daily mean flight intensity during the pre-application period varied between 4 to 16 honey bees/m² in the 4 different tents. On the day of treatment before spray application the honey bee flight activity with 11 to 16 honey bees/m² in the different treatments was far above the required level of > 3 honey bees/m² in all treatments. For all assessments the numbers of forager bees in both Coragen treatments were very similar to the control group. On the contrary in the reference tunnel the foraging activity decreased to almost nil during the whole post-treatment period (Figure 3).

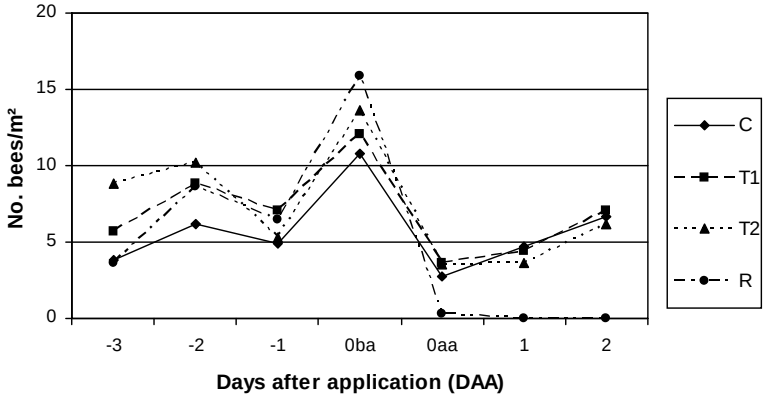

Figure 3 Honey bee flight intensity (number of honey bee/m²) in the control (C), Coragen at 60 g chlorantraniliprole/ha applied during honey bee flight (T1) or after daily bee flight (T2) and toxic reference treatment (dimethoate) (R) prior to and after spray application in winter wheat (sprayed daily with sugar solution to simulate honey dew) in France, 2006. (0ba = evaluation on the day of treatment shortly before application; 0aa = evaluation on the day of treatment after application)

The daily numbers of dead honey bee were homogenous and decreased from Day-3 to Day0 before application. The application of the toxic reference (400 g dimethoate/ha) induced a high peak mortality the day after spray application proving the sensitivity of the test system, while the numbers of dead forager bees in both Coragen treatments and in the control stayed all at about the same level as in the pre-treatment period (Figure 4). The two colony assessments before and after application did not show any significant changes due to Coragen applied during or after bee flight relative to the control. No symptoms of poisoning or abnormal behaviour were recorded during the whole trial period in any of the treatment groups, i.e. Coragen treatment groups relative to the control.

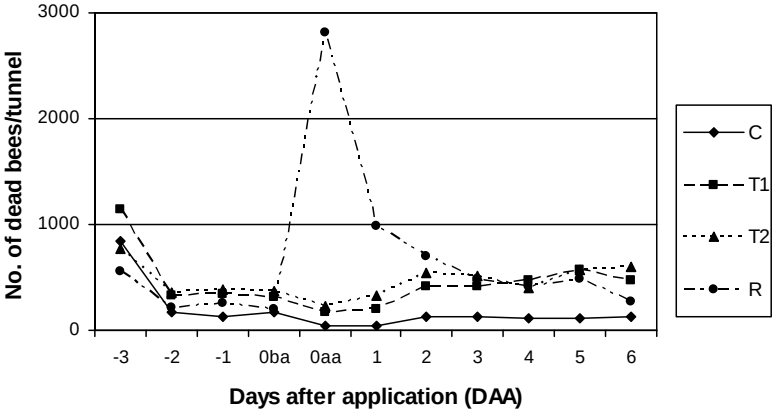

Figure 4 Number of dead honey bees per tunnel and day in the control (C), Coragen at 60 g chlorantraniliprole/ha applied during honey bee flight (T1) or after daily bee flight (T2) and toxic reference treatment (dimethoate) (R) prior to and after spray application in winter wheat (sprayed daily with sugar solution to simulate honey dew) in France, 2006. (0ba = evaluation on the day of treatment shortly before application; 0aa = evaluation on the day of treatment after application)

In all six semi-field honey bee tunnel trials following the CEB 230 design – either conducted with flowering *Phacelia* or wheat as crop – comparable levels of honey bee mortality were determined for the Coragen treatments (sprayed during honey bee foraging activity or outside of flight activity) and the control group. Also, there were no obvious differences found between the control group and the two Coragen treatment groups regarding flight intensity, behaviour, colony strength or presence of queen, eggs, larvae or pupae.

Results of semi-field tunnel honey bee test to quantify residue in bee matrices via systemic uptake from the soil

Chlorantraniliprole residue concentrations were determined in nectar and pollen of *P. tanacetifolia*, which was grown in soil treated with chlorantraniliprole, and in bee wax produced by honey bees foraging on the exposed plants. Residue concentrations in pollen, nectar and wax were determined following an application with chlorantraniliprole onto honey bees foraging on flowering *P. tanacetifolia* plants. Results are summarized in Table 3. Chlorantraniliprole residue concentrations in pollen and nectar foraged from plants grown in chlorantraniliprole pre-treated soil were found only in samples taken from bee legs and the honey stomach of forager bees collected in front of the hive and were significantly lower than residues resulting from direct spray application at 60 g chlorantraniliprole/ha. No chlorantraniliprole residues have been determined inside the hives of honey bees foraging on plants growing on chlorantraniliprole pre-treated soil, indicating that honey bees are not markedly exposed to systemic residues of chlorantraniliprole in plants.

Table 3 Maximum chlorantraniliprole residues (mg/kg) determined in nectar (from bee stomach content) and pollen (from honey bee legs) sampled from forager honey bees (outside hive) and determined in nectar, pollen and wax from honey bee combs inside honey bee hives from colonies kept in *Phacelia* control tunnels (n = 2) and, *Phacelia* tunnels following soil application at sowing with chlorantraniliprole at 253.6 g chlorantraniliprole/ha and following spray application at 60 g chlorantraniliprole/ha during honey bee foraging activity in flowering *Phacelia*.

		Forager bees - outside hive		Comb samples - inside hive		
Treatment	**DAA**	**Nectar**	**Pollen**	**Nectar**	**Pollen**	**Wax**
				(mg chlorantraniliprole/kg)		
Control	-1	**	**	**	*/**	**
	+1	**	**	**	*/**	**
	+3	**	**	ns	ns	ns
	+7	**	**	**	**	*/**
Soil application	-1	*	*	**	**	**
	+1	*	*	**	**	**
	+3	0.0032	0.0010	ns	ns	ns
	+7	*	0.0018	**	**	**
Spray onto foraging bees in flowering *Phacelia*	-1	**	**	**	**	**
	+1	0.0330	2.6010	0.0472	2.8630	0.0105
	+3	0.0096	0.7633	ns	ns	ns
	+7	0.0036	0.2643	0.0013	0.1080	0.0757

*: < LOQ = Limit of Quantification = 0.001 mg chlorantraniliprole/kg; **: < LOD = Limit of Detection = 0.0003 mg chlorantraniliprole/kg, ns: not sampled

Results on bumble testing under commercial greenhouse conditions

The mean numbers of dead larvae, pupae and adult bumble bees per day and hive in the 4 subplots before application were 1.6 in the control treatment and 1.6, 1.4, 2.8 and 1.3 in the Altacor treatments T1, T2, T3 and T4, respectively. From DAA0 until DAA+7 after the application in C and T1 the mean number of dead larvae, pupae and adult bumble bees per day and hive of the four replicates, respectively, was between 0.6 and 1.0 in all treatment groups without any differences between the treated and the control plots (Figure 5).

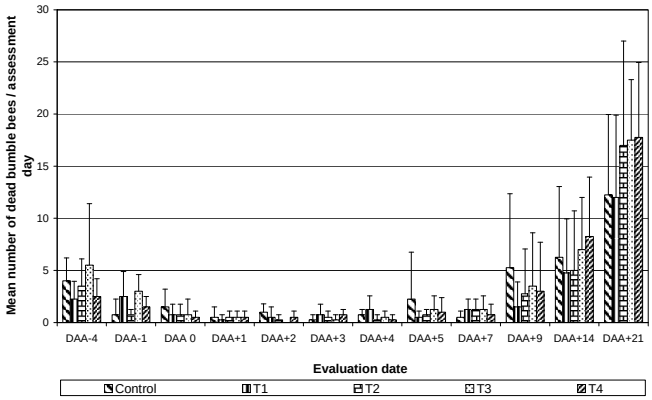

Figure 5 Mean number of dead bumble bees per day (adult workers and larvae) (± SD) collected in the field, in front and inside the four colonies of the control and the Altacor treatments T1 to T4. (T1 = Altacor applied during foraging activity of the bumble bees, T2, T3 and T4 = Altacor applied 24 h, 48 h and 72 h before opening the hives, respectively; T2, T3 and T4 were applied with closed bumble bee hives and no bumble bees in the plots.)

From DAA+9 mortality increased in all treatment groups in parallel due to the increasing strength of the colonies and the decreasing food availability in the greenhouse, and was mainly caused by larval death. During the entire assessment period after application the mean number of dead bumble bees per day and hive in the 4 subplots was 3.1 in the control treatment, 2.4, 2.9, 3.3 and 3.3 in the Altacor treatments T1, T2, T3 and T4, respectively. No statistically significant differences between the control and any of the Altacor treatment groups were calculated (Dunnett's t-test and Bonferroni U (Holms) Exact test, two-sided; $p \leq 0.05$). In all treatment groups the bumble bees started immediately (on the day after set-up in the greenhouse) pollinating the crop and leaving on flowers visited so called "bite marks" and a continuous increase of the pollination activity (increase of the number of points) was observed during the course of the study from DAA-4 up to DAA+2 when pollinating activity approached a maximum (Figure 6).

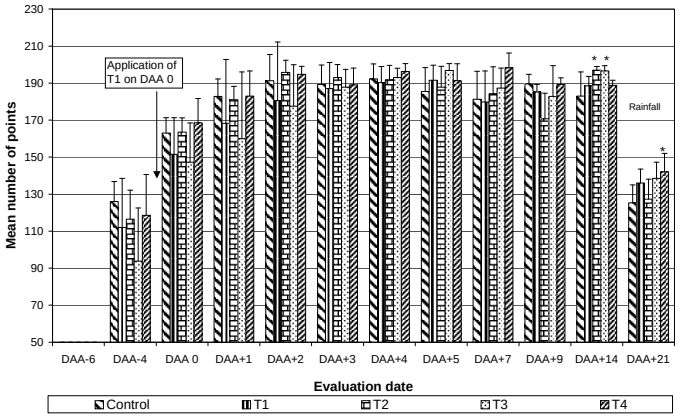

Figure 6 Foraging activity of bumble bees given as mean number of points[#] in the control and the Altacor treatments T1 to T4. (T1 = Altacor applied during foraging activity of the bumble bees, T2, T3 and T4 = Altacor applied 24 h, 48 h and 72 h before opening the hives, respectively; T2, T3 and T4 were applied with closed bumble bee hives and no bumble bees in the plots.). (* = significantly higher than the control (Dunnetts t-Test ($p \leq 0.05$, two-sided)) ([#] = Category 1: no bite mark = 1 point; Category 2: 1-3 bite marks/flower = 2 points; Category 3: > 3 bite marks/Flower = 3 points; category 4: brown Pistil = 4 points.)

From DAA+2 the pollinating activity remained at high level until end of the exposure period on DAA+21. Foraging activity was statistically significantly higher than in the control group on DAA+14 in the Altacor treatment T2 and T3, and on DAA+21 in T4 (Dunnett's t-test, two-sided; $p \leq 0.05$). On DAA+21 foraging activity was relative low, but comparable in all treatment groups due to bad weather conditions.

The mean sugar solution uptake of the bumble bees was similar in the control treatment and in the Altacor treatments T1, T2, and slightly higher in T3 and T4 (Figure 7). The mean sugar solution consumption of the bumble bees from the set-up of the colonies until the last day of exposure was 580 g in the control and 584 g, 593 g, 670 g and 708 g in the Altacor treatment T1, T2, T3 and T4, respectively. No statistically significant differences between the control and any of the Altacor treatment groups were calculated (Dunnett's t-test, Bonferroni U (Holms) Exact test, Welch Bonferroni Holms corrected, two-sided, $p \leq 0.05$).

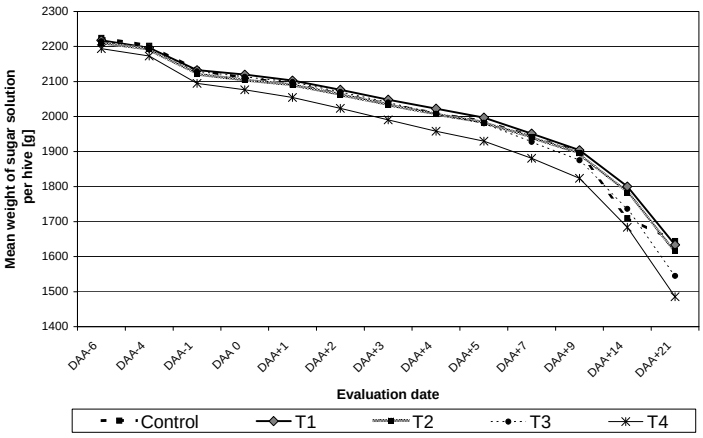

Figure 7 Mean weight of the sugar solution bags of the four colonies of the control and the Altacor treatments T1 to T4. (T1 = Altacor applied during foraging activity of the bumble bees, T2, T3 and T4 = Altacor applied 24 h, 48 h and 72 h before opening the hives, respectively; T2, T3 and T4 were applied with closed bumble bee hives and no bumble bees in the plots.)

During the assessment period from DAA-6 up to DAA0 slight fluctuations in the weight of the colonies were observed in all treatment groups. From DAA0 to the last assessment date on DAA+21 the mean weight in the colonies of the treatment groups increased clearly (Figure 8). In view of the total observation period from DAA-6 until DAA+21 the colonies of all treatment groups increased their mean weight, i.e. the colonies of the control treatment by 97 g and 109 g, 109 g, 127 g and 131 g in the Altacor treatments T1, T2, T3 and T4, respectively. No statistically significant differences between the control and any of the chlorantraniliprole treatment groups were calculated (Dunnett's t-test, Bonferroni U (Holms) Exact test, two-sided, $p \leq 0.05$).

When the final brood assessment was carried out 22 days after the start of exposure all hives were in the process of disposing their original living old queens which had been in the hives since the start of the study. Additionally, one colony of C, two colonies of T1, two colonies of T2, three colonies of T3 and three colonies of T4 had young newly hatched queens, respectively. Two colonies of the control, one colony of T2 and one colony of T4 had unhatched queen pupae. In one colony of the control and one of T3 no eggs were found. In all other colonies the presence of the queen, eggs, larval stages and pupae showed that the colonies were in good condition. The mean number of worker bees in the colonies of the treatment groups at the final brood assessment was 100 in the control treatment and 119, 129, 159, and 153 in the Altacor treatments T1, T2, T3 and T4, respectively. No abnormal differences in brood development, which could be attributed to the influence of chlorantraniliprole were observed between the control and the Altacor treatments.

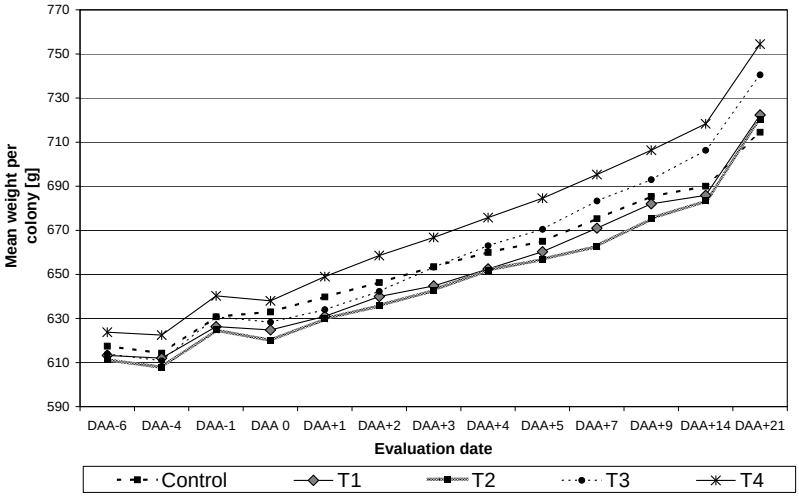

Figure 8 Mean weight of the four bumble bee colonies of the control and the Altacor treatments T1 to T4. (T1 = Altacor applied during foraging activity of the bumble bees, T2, T3 and T4 = Altacor applied 24 h, 48 h and 72 h before opening the hives, respectively; T2, T3 and T4 were applied with closed bumble bee hives and no bumble bees in the plots.)

Discussion

Chlorantraniliprole exhibited low intrinsic toxicity for honey bees. This is in line with findings of low sensitivity for other hymenopteran species, e.g. the parasitic wasp, *Aphidius rhopalosiphi*, for which the LR50 and ER50 values – based on mortality and reproduction – were both > 750 g chlorantraniliprole/ha on inert substrate (glass plates) for Coragen and Altacor[4]. When the active substance chlorantraniliprole was tested up the maximum water solubility of 1 mg chlorantraniliprole/L survival and behaviour of treated honey bees were unaffected. The same observations and lack of any lethal or sub-lethal effects were made for the formulated product Altacor, the wet-able granule, up to maximum tested rates (oral and contact) of >119.2 and 100 µg chlorantraniliprole/bee, respectively. When chlorantraniliprole was applied in combination with the organic solvent, acetone, sub-lethal effects were observed. Also for the liquid formulation, Coragen, some honey bees showed sub-lethal effects, but only at higher dosages tested. From these observations it can be concluded that chlorantraniliprole is unlikely to reach and affect the target sites, ryanodine receptors, in honey bees, when dissolved in watery solutions. The calculated hazard quotient (HQ) values for both formulations Coragen and Altacor were all <1 and far below the EU-relevant trigger value of 50 predicting high margins of safety for honey bees in flowering crops. Also for application rates of chlorantraniliprole above the EU-intended uses the HQ values will remain below the EU HQ trigger of 50 indicating low risk for honey bees for the world-wide intended uses of chlorantraniliprole.

The prediction of low risk for honey bees due to the uses of chlorantraniliprole were confirmed in numerous semi-field tunnel tests with Coragen conducted under worst-case exposure conditions in various locations in Germany, France and Spain and in different years. In these tests it was found that the spray application of chlorantraniliprole performed during full foraging activity or exposure to spray deposits (treatment outside foraging) in flowering crops (*Phacelia*) or crops bee-attractive due to honey dew (simulated via daily sprays of sugar solutions) did not have any effects regarding all parameters assessed, i.e. mortality, foraging activity, behaviour or condition of the colonies and development of honey bee brood assessed for up to 28 days after treatment relative to water treated controls for rates up to 60 g chlorantraniliprole/ha. No negative

effects were found in an early *Phacelia* tunnel screening test sprayed at 75 g chlorantraniliprole/ha during foraging activity of honey bees (DuPont, unpublished).

Exposure to chlorantraniliprole residues from carry over in soil after applications at planting to the nectar or pollen in future flowering crops was much lower than residues from direct exposure of honey bees via direct spray application. No quantifiable amounts of chlorantraniliprole residues were found inside the hives via worst-case soil dosing and simulation of a long-term plateau concentration resulting from continuous maximum use over multiple years.

Chlorantraniliprole was compatible with bumble bees as crop pollinators in greenhouses. Altacor when applied during foraging activity or 24 h, 48 h or 72 h before opening of the hives of the bumble bee, *B. terrestris*, at a rate of 40 g chlorantraniliprole/ha (maximum recommended rate) and an application volume of 1000 L per ha did not have any effects regarding all parameters assessed, i.e. mortality, foraging activity, condition of colonies and development of bumble bee brood relative to the water treated control. The low toxicity of chlorantraniliprole was also confirmed for another bumble bee species, *Bombus impatiens* Cresson, an important indigenous pollinator in North America[10]. Adult *B. impatiens* worker bees didn't shown any increased mortality (0% mortality corrected for control) when exposed via direct spray contact (Potter tower) to spray solutions (19:1 acetone: olive oil solution) containing 0.001, 0.01 and 0.1 % chlorantraniliprole, while other insecticides significantly increased mortality to levels >80% mortality tested at the same three concentrations (imidacloprid) or at the 2 hightest tested concentrations of 0.01 and 0.1 % (metaflumizone and abamectin). Chlorantraniliprole at up to 900 ppm did not affect survival, infectivity, and reproduction of the entompathogenic nematode, *Heterorhabditis bateriphora*, offering e.g. a highly effective option for remedial white grub control in greenhouses[11].

Conclusions

Chlorantraniliprole (DuPont™ Rynaxypyr) and its formulated products, Coragen and Altacor demonstrated low intrinsic toxicity for honey bees and bumblebees. In worst-case tunnel and greenhouse trials no significant effects on pollinating bees were found, even when bees were directly over-sprayed during foraging activity. This indicates a high margin of safety for honey bees and bumble bees for the uses of chlorantraniliprole and its formulated products, Coragen and Altacor, in flowering crops and in succeeding crops. As chlorantraniliprole has proven to have negligible effects on numerous beneficial non-target arthropod species or to have a rather low and transient impact on some beneficial species, too, Coragen and Altacor provide excellent tools for integrated pest management (IPM) programmes. In line with Good Agricultural Practice and to avoid unnecessary contamination of pollinators spray applications should always be made when pollinators are not foraging or after daily bee flight.

Acknowledgements

Sincere thanks to all co-operators, i.e. those performing the honey bee and bumble bee studies, and contributing to the development of chlorantraniliprole.

References

1. Cordova D, Benner EA, Sacher MD Rauh JJ, Sopa JS, Lahm GP, Selby TP, Stevenson TM, Flexner L, Gutteridge S, Rhoades DF, Wu L, Smith RM, Tao Y, Anthranilic diamides: A new class of insecticides with a novel mode of action, ryanodine receptor activation. *Pestic Biochem Phys* **84**: 196-214 (2006).
2. Lahm GP, Stevenson TM, Selby TP, Freudenberger JH, Cordova D, Flexner L, Bellin CA, Dubas CM, Smith BK, Hughes KA, Hollingshaus JG, Clark CE, Benner EA. Rynaxypyr™: A new insecticide anthranilic diamide that acts as a potent and selective ryanodine receptor activator. *Bioorg Med Chem Lett* **17**: 6274-6279 (2007).
3. Bassi A, Alber R, Wiles JA, Rison JL, Frost NM, Marmor FW, Marcon PC, Chlorantraniliprole: a novel anthranilic diamide insecticide. *Proceedings of XVI International Plant Protection Congress 2007*, **Vol. 1**: 52-59 (2007).

4. Dinter A, Brugger K, Bassi A, Frost N-M, Woodward M (2008): Chlorantraniliprole (DPX-E2Y45, DuPont™ Rynaxypyr, Coragen and Altacor insecticide) - a novel anthranilic diamide insecticide - demonstrating low toxicity and low risk for beneficial insects and predatory mites. WG Pesticides and Beneficial Organisms, Proceedings of the Meeting at Berlin, Germany, 10th - 12th October 2007, Editors: Heidrun Vogt, Jean-Pierre Jansen, Elisa Vinuela & Pilar Medina. *IOBC/wprs Bull* **35**: 128-135 (2008).
5. OECD, Guideline for testing of chemicals. Honeybees. Acute oral toxicity test. *OECD* **213**: 8 p. (1998).
6. OECD, Guideline for testing of chemicals. Honeybees. Acute contact toxicity test. *OECD* **214**: 7 p. (1998).
7. EPPO, Guideline for the efficacy evaluation of plant protection products – Side effects on honeybees. OEPP/EPPO, PP 1/170 (3) update 2000, 19 - 23 (2001).
8. CEB, Commission des Essais Biologiques - Méthode d'évaluation des effets de toxicité aiguë et a court terme des préparations phytopharmaceutiques sur l'abeille domestique (*Apis mellifera* L.). *ANPP*, méthode no. 230: 8 – 21 (2003).
9. Barrett KL, Grandy N, Harrison EG, Hassan S, Oomen P (eds.), Guidance Document on Regulatory Testing Procedures for Pesticides with Non-Target Arthropods. From the ESCORT Workshop (European Standard Characteristics of Beneficial Regulatory Testing); Wageningen, Holland, 28 - 30 March, 1994. 51pp (1994)
10. Gradish AE, Scott Dupree CD, Shipp L, Harris CR, Ferguson G, The effect of reduced risk pesticides for use in greenhouse vegetable production on bumble bee (*Bombus impatiens* Cresson). WG Integrated Control in Protected Crops, Temperate Climate, Proceedings of the Meeting at Sint Michielsgestel (The Netherlands), 21-25 April, 2008. Edited by: Annie Enkegaard. *IOBC/wprs Bull* **32**: 67-70 (2008).
11. Koppenhöfer AM, Fuzy, EM, Effect of the anthranilic diamide insecticide, chloranthraniliprole, on *Heterorhabditis bacteriophora* (Rhabditida, Heterorhabditidae) efficacy against white grubs (Coleoptera, Scarabaeidae). *Biol Control* **45**: 93-102 (2008).

Honey bee brood ring-test: method for testing pesticide toxicity on honeybee brood in laboratory conditions

Pierrick Aupinel[1*], Dominique Fortini[1], Bruno Michaud[1], Piotr Medrzycki[2], Erica Padovani[2], Dunja Przygoda[3], Christian Maus[3], Jean-Daniel Charriere[4], Verena Kilchenmann[4], Ulrike Riessberger-Galle[5], Jutta Juliane Vollmann[5], Lukas Jeker[6], Martina Janke[7], Jean-François Odoux[1], Jean-Noël Tasei[1]

[1]Unité expérimentale d'entomologie, INRA, Le Magneraud, BP 52, 17700 Surgères, France
[2]Consiglio per la Ricerca e la Sperimentazione in Agricoltura, Unità di Ricerca di Apicoltura e Bachicoltura, Via di Saliceto 80, 40128 Bologna, Italia
[3]Bayer CropScience AG, Ecotoxicology, 6620, Alfred-Nobel-Str. 50, 40789 Monheim, Germany
[4]Agroscope Liebefeld-Posieux ALP, Schwarzenburgstrasse 163, CH - 3003 Berne Switzerland
[5]Institut für Zoologie, Abteilung Stoffwechselphysiologie und Verhalten, Universitätsplatz 2, A-8010 Graz, Austria.
[6]Harlan Laboratories Ltd. Zegliwed 1 CH-4452 Itingen Switzerland
[7]LAVES Laves Institut für bienenkunde Celle, Germany
*Tel.: +33(0)546683002, fax: +33(0)546683062, e-mail: Pierrick.Aupinel@magneraud.inra.fr

Abstract

The Experimental unit of entomology (INRA, France) developed a new *in vitro* method to assess effects of pesticides on honey bee larvae. The method consists in rearing bee larvae in plastic cells. The larvae are fed with diet containing 50% of fresh royal jelly and 50% of an aqueous sugar and yeast extract solution, and reared in an incubator at 35 °C and 96% relative humidity. According to that method, 9 tests (7 in 2008 and 2 in 2005) were carried out in 7 laboratories and different countries. The objective of these trials was to assess the LD_{50} for dimethoate 48 hours after an acute exposure.

The LD_{50} values ranged from 1.5 µg a.i./larva to 8.8 µg a.i./larva, with 2 tests with particularly high values (5.0 and 8.8 µg a.i./larva). In 7 tests, these values ranged from 1.5 µg a.i./larva to 3.1 µg a.i./larva. Such variability may be due to the colony origin, the season and larva heterogeneity at grafting. Solutions are proposed to improve the method through the continuation of the ring test.

Keywords**:** *Apis mellifera*, brood, *in vitro* test, dimethoate